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Evaluation of promoters and visual markers for transformation of eastern white pine

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Abstract. This report serves to evaluate possible promoters for use in the production of transgenic eastern white pine (*Pinus strobus* L.). Embryogenic cultures of eastern white pine were bombarded with gold particles coated separately with a variety of gene constructs containing the *UidA* β-glucuronidase (GUS) or green fluorescent protein (GFP) reporter gene. Transient expression of the *UidA* gene, driven by a novel algal virus adenine methyl transferase gene promoter, as well as five other promoters used in angiosperm transformation, were evaluated. The maize alcohol dehydrogenase promoter was not effective in eastern white pine cultures. The construct with the doubled Cauliflower Mosaic Virus 35S promoter plus Alfalfa Mosaic Virus enhancer showed the highest levels of expression. GUS expression was detected within 24 hours, but decreased after 5 days and was not detectable 15 days after bombardment. Expression of GUS activity was recorded mainly in somatic embryonal heads of various stages of development and occasionally in suspensor cells. Similar to GUS expression, modified green fluorescent protein (GFP) was detected in the embryonal head cells 24 hours after bombardment. GFP-expressing suspensor cells were both more infrequent and difficult to detect, as their highly vacuolate nature rendered the GFP presence less visible against the yellow background autofluorescence.

Key words: biolistics, GFP,-GUS, *Pinus strobus* L., transient gene expression

Abbreviations: benzylaminopurine, BAP; cauliflower mosaic virus 35S promoter, 35S; 2,4-dichlorophenoxyacetic acid, 2,4-D; green fluorescent protein, GFP; β-glucuronidase, GUS; plant growth regulator, PGR

Introduction

Microprojectile-mediated DNA transfer has been shown to be effective for a variety of woody angiosperms and gymnosperms. Gymnosperm tissues used as biolistic targets have included cotyledons (Stomp et al. 1991), vegetative

buds and bud-derived calluses (Aronen et al. 1994), mature pollen (Hay et al. 1994; Li et al. 1994), xylem (Loopstra et al. 1992), and somatic embryogenic cultures (e.g. Duchesne and Charest 1991; Robertson et al. 1992; Bommineni et al. 1994; Walter et al. 1994; Clapham et al. 1995). Expression has been most commonly transient, for several possible reasons (Clapham et al. 1995); however, stable incorporation has been documented in a few conifer species (e.g. Ellis et al. 1993; Charest et al. 1996).

Surveys are necessary for finding promoters appropriate during the selection process as well as for driving successful expression of the gene of interest in the plant. Early reports of conifer transformation utilized promoters that were most effective in dicotyledonous systems, especially the Cauliflower Mosaic Virus 35S promoter or modified versions (e.g. Stomp et al. 1991; Campbell et al. 1992; Shin et al. 1994). Monocot promoters have also been investigated, and have shown varying degrees of effectiveness (Ellis et al. 1991 vs. Loopstra et al. 1992; Walter et al. 1994).

This study evaluates several promoters, including an algal virus gene promoter not previously reported for use in the genetic manipulation of tree species. In addition, two commonly used visual markers, Green Fluorescent Protein (GFP) and β-glucuronidase (GUS), were compared for their utility in eastern white pine transformation.

Materials and methods

Preparation of embryogenic tissues for bombardment

Embryogenic cultures of eastern white pine, *Pinus strobus* L., were initiated and maintained for 2 years as described by Kaul (1995). Immature seeds were surface-sterilized in 10% Clorox™ for 10 min and then rinsed three times in sterile water. Excised megagametophytes were soaked in 5% Clorox™ for 5 min, rinsed and then transferred to DCR medium (Becwar et al. 1990) containing 12 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 4 μM benzylaminopurine [BAP], and solidified with 0.2% Gelrite (Sigma, St. Louis, MO). As the cones came from open-pollinated trees, a culture arising from each megagametophyte was designated as an individual genotype line (e.g. WP/B2, WP/7) and maintained separately on gelled medium. Embryogenic cultures were separated from their respective megagametophytes after 8–10 weeks and sub-cultured at two or three week intervals for 4 months. They were then transferred to Brown and Lawrence medium (Brown and Lawrence 1968) containing 73 mM (2.5%) sucrose, 0.65% agar (Sigma, St. Louis, MO) and modified to contain L-glutamine (10 mM) as the

sole source of amino nitrogen. The PGRs 2,4-D (8 μM) and BAP (4 μM) were added, and the pH was adjusted to 5.8. All cultures were incubated in the dark at 20–22 °C, and subcultured every three weeks. Embryogenic cultures were transferred to fresh medium 5–7 days prior to bombardment and spread uniformly to a circle of approximately 2–3 cm in diameter. One plate per treatment was used as a nonbombarded control. For the genotype expression experiment, matched replicate cultures of two lines, identified as WP/B2 and WP/7, respectively, were prepared for each bombardment using common pools of DNA-coated particles, as was a nonbombarded control. Three diverse constructs were utilized in this experiment.

Microprojectile bombardment

Procedures used for 1 μm gold particle microprojectile preparation and coating with DNA were according to Heiser (1992). Each culture was bombarded twice with 1 μg of construct DNA per macrocarrier disk. Each plate was rotated 90° between bombardments. Bombardment with the Biolistic™ particle delivery system PDS-1000/He (DuPont, Wilmington, DE) employed a rupture disc pressure of 1100 psi and a sample distance of 5 cm. Following bombardment, the cultures were incubated in petri dishes in the dark at 20–22 °C.

Constructs

The following constructs, all containing the GUS reporter gene (Jefferson et al. 1987), were used (Figure 1): 1) pAHC25, driven by the maize ubiquitin *Ubi1* promoter and first intron (Christensen et al. 1992), provided by Dr. Peter Quail, USDA/ARS, Plant Gene Expression Center, Albany, CA; 2) pAI_1GUS_nDy10s, driven by the maize alcohol dehydrogenase (*adh1*) promoter and first intron, provided by Dr. Ann Blechl, USDA/ARS, Crop Improvement and Utilization Research Unit, Albany, CA; 3) pAMTGUS25, driven by the *Chlorella* virus adenine methyl transferase gene promoter (Mitra and Higgins 1994) plus *adh1* first intron, provided by Dr. Amit Mitra, Department of Plant Pathology, University of Nebraska, Lincoln, NE; 4a) pMON752, driven by a chimeric double 35S promoter plus *adh1* intron 1, and 4b) pMON18350, driven by a chimeric double 35S promoter, provided by Dr. Tim Conner and Dr. M.E. Fromm, Monsanto Co., St. Louis, MO and 5) pBI426, driven by a double 35S promoter plus an alfalfa mosaic virus (AMV) enhancer sequence (Datla et al. 1991), provided by Dr. Raju Datla, National Research Council Canada, Plant Biotechnology Institute, Saskatoon, Saskatchewan. The plasmid pHBT-SGFP-TYG-NOS, containing a synthetic GFP

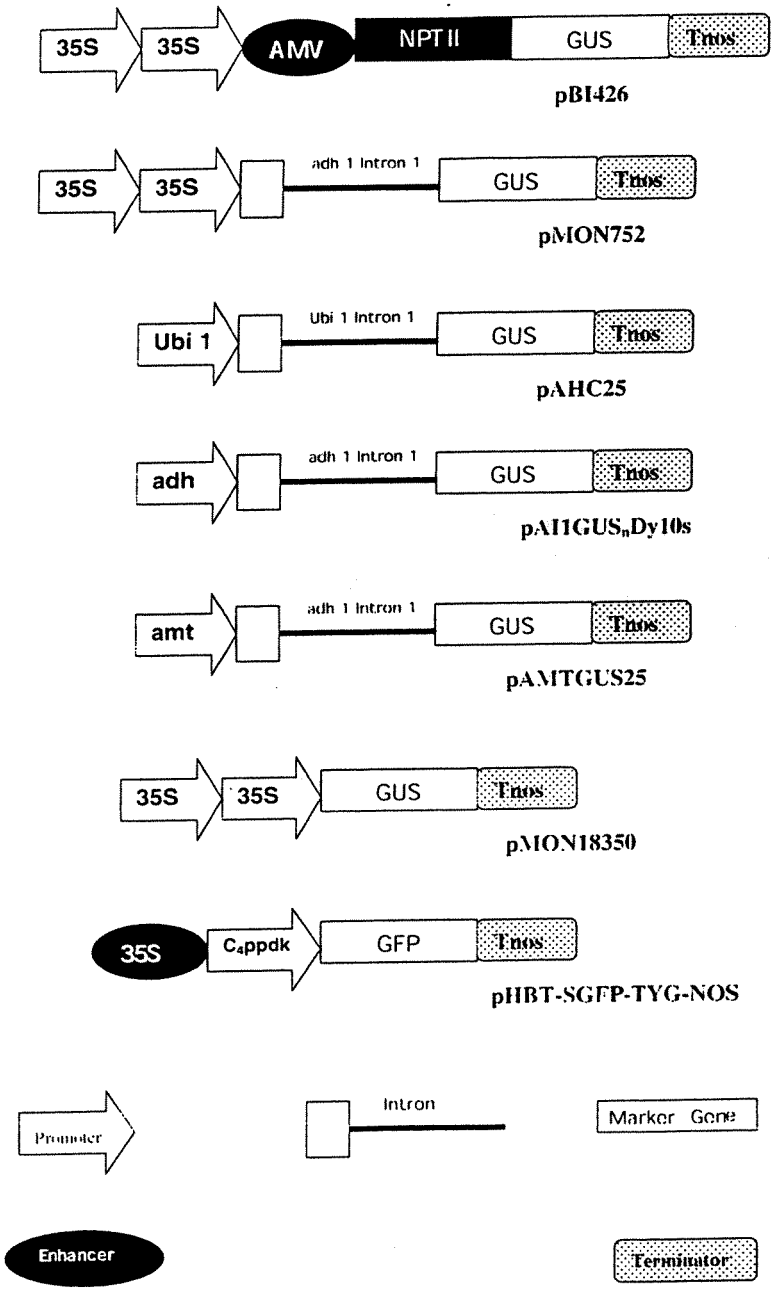

Figure 1. Promoter constructs used to study visual marker expression in eastern white pine embryogenic cultures. Regions are not to scale and are for illustrative purposes only. The "enhancer" is transcriptional in the case of the 35S enhancer and translational in the case of the alfalfa mosaic virus (AMV) enhancer.

mutant gene driven by a hybrid 35S enhancer – maize C_4 pyruvate *ortho*-phosphate dikinase 1 (*ppdk1*) promoter (Sheen 1993), was provided by Dr. J. Sheen, Department of Molecular Biology, Massachusetts General Hospital, Boston, MA.

Visual marker assays

1. Histochemical GUS assay
Samples of approximately one quarter of the target circle were assayed according to Jefferson et al. (1987) 1 day after bombardment. In a smaller study, select calli bombarded with two promoter constructs were sampled 5 and 15 days after bombardment. Both isolated individual cells and clusters of blue-stained cells were counted as single GUS-expressing sites.

2. GFP fluorescence assay
Single quadrants of those cultures bombarded with the GFP construct were assayed using a fluorescent microscope (Olympus America, Melville, NY). Samples were excited by blue light (λ_{max} = 490 nm) and observed via an Olympus BP490 filter cube. Numbers of green fluorescing cells were counted for only samples collected 1 day after bombardment.

Statistics

Analysis of variance and Duncan's Multiple Range Tests of GUS-positive foci ("blue spot") frequency were performed using the SAS System for Windows, Version 6.12 (SAS Institute, Cary, NC). GUS expression driven by the different promoters was analyzed in a completely randomized design with unequal replications. Genotype-dependent expression (WP/B2 vs. WP/7) was analyzed using a randomized complete block design.

Results and discussion

GUS expression

All embryogenic cultures expressed GUS activity 24 hours after bombardment. Photomicroscopy of cells showed both multiple GUS blue-colored "hits" on single embryos, and numerous isolated individual or small clumps of cells showing the same characteristic blue color (Figures 2A, B). Occasionally suspensor cells expressed GUS (Figure 2A). Twenty-four-hour assays showed intense GUS activity in single cells as well as in small clusters of 3–6 cells, probably arising from mitosis after successful bombardment and

nuclear incorporation. In contrast, a light blue color frequently extended from a transformed cell to the neighboring cells, probably due to GUS gene product diffusion. Similar observations were also reported in bombarded cotyledons of loblolly pine (*Pinus taeda*) (Stomp et al. 1991).

All promoters tested showed some level of GUS expression, based on numbers of blue-colored sites (Figure 3), while endogenous activity was never detected in any bombarded or nonbombarded control. Based on the average number of GUS-expressing sites, the most effective of those promoters tested in white pine embryogenic tissue was the double-35S plus AMV enhancer (pBI426). This was followed by the *Ubi1* promoter + first intron (pAHC25) and the double 35S promoter (pMON18350) which was not significantly different from the *amt* promoter plus *adh1* intron 1 (pAMTGUS25) and the double 35S plus *adh1* intron 1 (pMON752) which was significantly different from the *adh1* promoter + first intron (pAI_1GUS_nDy10s) (Figure 3). The *adh* promoter exhibited extremely low numbers of GUS foci as was also seen in bombarded white spruce (*Picea glauca*) embryogenic calluses (Ellis et al. 1991). Several double 35S promoters and their derivatives have been tested previously in other conifer species (e.g. Bekkaoui et al. 1990; Newton et al. 1992; Charest et al. 1993; Bommineni et al. 1994; Walter et al. 1994). In general 35S and double 35S types were characterized by medium to strong expression, similar to our study, with double 35S types outperforming the single promoter constructs. A dicot (sunflower) polyubiquitin promoter achieved the highest GUS expression in pollen of Norway spruce (*Picea abies*) and Scots pine (*Pinus sylvestris*) (Haggman et al. 1997). Although the functional promoter region is organized differently, the maize ubiquitin promoter induced similarly high levels of GUS expression in our system. Apparently the presence of a 5′ intron has little effect on gene expression in gymnospermous systems.

The novel promoter, *amt*, is an 851 bp upstream region from a *Chlorella* virus methyl transferase gene that has been shown to be effective for expression in transformants of a small number of both monocots and dicots (Mitra et al. 1994). No applications of this promoter to tree transformation have been reported in the literature.

Typically, transient GUS activity decreased within 5 days after bombardment and whole tissue assays showed no GUS activity after 15 days (Table 1). There was no indication of plasmid gene incorporation without chemical (antibiotic, herbicide) selection, as has been seen in transformations of other plant species (McCabe and Martinell 1993).

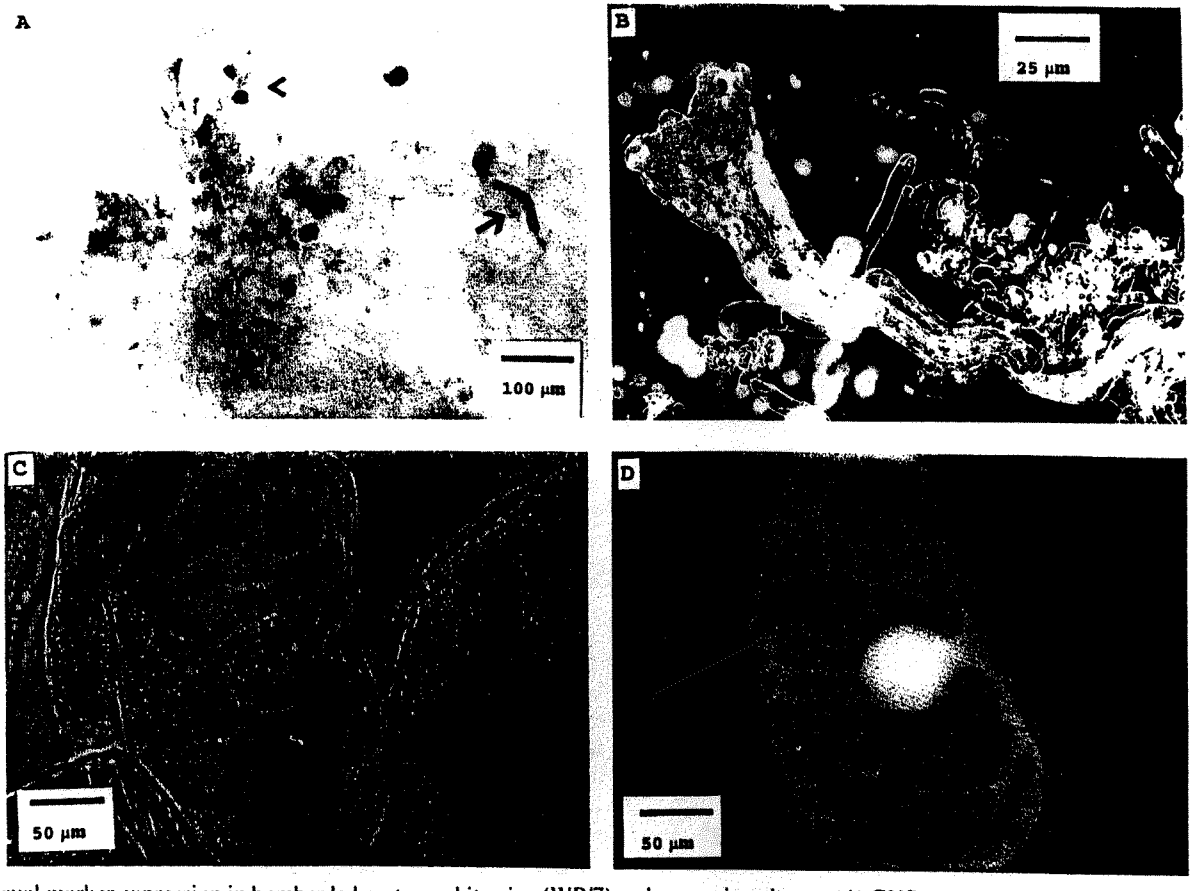

Figure 2. Visual marker expression in bombarded eastern white pine (WP/7) embryogenic cultures. (*A*) GUS expression in both suspensor cell (arrow) and embryonal heads (arrowhead) of eastern white pine embryogenic callus, (*B*) Two bombarded and transformed GUS-expressing cells in an early stage eastern white pine somatic embryo, and (*C, D*) GFP expression in suspensor cell of eastern white pine embryogenic callus. (*C*) Bright field image. (*D*) Fluorescent image of the same cell. Note presence of nearby non-fluorescing cells. Assayed one day after bombardment with pMON752 (*A, B*) or pHBT-SGFP-TYG-NOS (*C, D*).

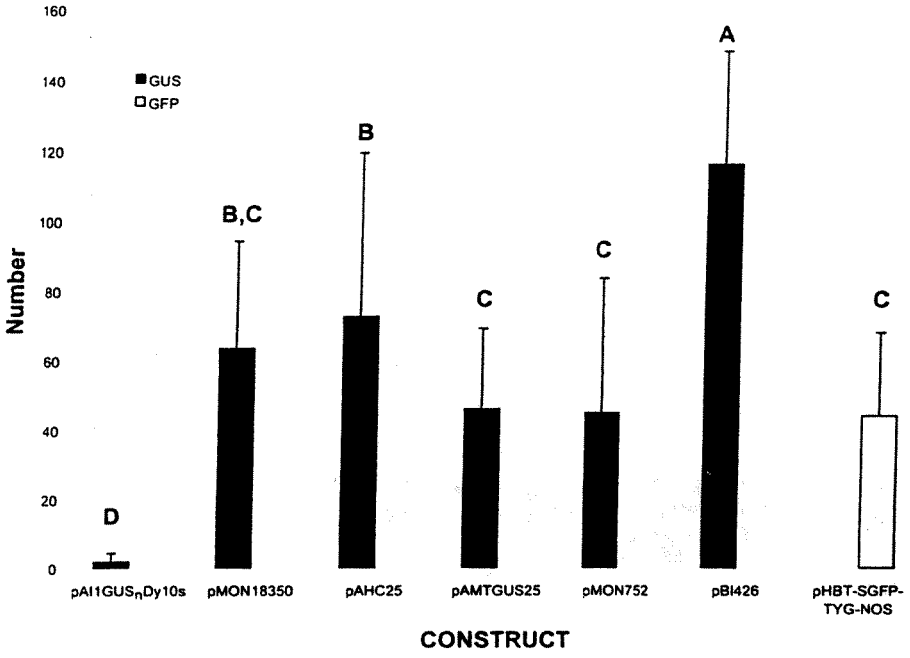

Figure 3. Comparison of transient GUS and GFP expression driven by different promoters in genotype WP/7 eastern white pine callus. Number is average number of blue spots (solid bars) or green fluorescing cells (open bar) counted per quarter of embryonal culture mass assayed one day after bombardment; error bars represent one standard deviation. Means with the same letter are not significantly ($P = 0.05$) different according to Duncan's Multiple Range Test.

Table 1. Representative differential transient GUS expression in embryonal cultures (WP/7) of eastern white pine (*Pinus strobus*).

Construct (promoter)	GUS expression[1]		
	Day 1	Day 5	Day 15
pAMTGUS25 (*amt* + *adh1* intron 1[2])	31.8 ± 9.2	6.1 ± 1.7	0.0 ± 0.0
pAHC25 (*Ubi1* + first intron)	84.0 + 19.9	23.7 + 10.6	0.0 + 0.0

[1] Average number (± standard deviation) of blue spots counted per quarter of embryonal culture mass assayed the listed days after bombardment.
[2] Promoters used in this study: *amt* – *Chlorella* virus adenine methyl transferase; *Ubi1* – maize ubiquitin 1.

Table 2a. Analysis of variance (ANOVA) for transient GUS expression in two eastern white pine genotypes bombarded with pAHC25 (*Ubi1* + intron), pMON18350 (double 35S) or pBI426 (double 35S + AMV enhancer).

Source	df	Mean squares
Replication	2	543.2 **NS**
Construct	2	16207.1****
Genotype	1	53792.0****
Construct × Genotype	2	296.2 **NS**
Error	64	426.1

NS; **** = not significant and significant at ($P = 0.0001$) level, respectively.

Table 2b. Effect of eastern white pine genotype on transient GUS expression after bombardment with pAHC25 (*Ubi1* + intron), pMON18350 (double 35S) or pBI426 (double 35S + AMV enhancer).

Genotype	N	Mean number of "hits"
WP/7	36	84.2 **A**
WP/B2	36	29.5 **B**

Means with the same letter are not significantly different ($P = 0.05$) according to Duncan's Multiple Range Test.

Genotype-dependent expression

Transient expression of the GUS reporter gene consistently varied between the two lines of eastern white pine (*Pinus strobus* L.) embryogenic cultures when either pBI426 or pAHC25 or pMON18350 were used for bombardment. Analysis of variance of data from the randomized complete block design revealed no significant Construct × Genotype interactions, but that only the construct and genotype were significant (Table 2a). GUS expression in the bombarded somatic embryo masses of WP/B2 was significantly different from that of bombarded WP/7 cultures, based on Duncan's Multiple Range Test (Table 2b).

This is the first report of genotype differences in transgene expression in eastern white pine tissues. Genotype variability in reporter gene expression has been noted in other conifer species. Variation of genotype response to bombarded GUS-containing plasmids has been noted in mature tissues (Aronen et al. 1995) and cultured cells derived from mature tissues (Aronen et al. 1994) of Scots pine (*Pinus sylvestris*). Embryogenic cell lines of *Picea mariana* also showed genotype variability when bombarded, with one family showing no GUS gene activity at all (Duchesne and Charest 1991).

Bombarded embryogenic suspensions of *Picea abies* had higher levels of GUS expression than embryogenic callus or zygotic embryos (Newton et al. 1992). Several lines of embryogenic cultures of *Larix laricina* were also bombarded with various GUS constructs resulting in varying expression levels (Klimaszewska et al. 1997).

The reason for the difference in transgene expression in WP/B2 vs. WP/7 must lie at some fundamental level, as GUS expression driven by both monocot and dicot promoters was similarly reduced. The cultures of both lines were morphologically similar in texture, color and state of development. Both cultures grew at similar rates and so there were no obvious differences to account for the variation in expression.

GFP expression

A construct using a modified green fluorescent protein (GFP) sequence (Chiu et al. 1996) was also employed to test the utility of GFP vs. GUS as a visual marker system for eastern white pine transformation. Embryogenic eastern white pine cells autofluoresce a bright yellow when excited with long UV wavelengths. In work reported here, GFP expression was detectable 24 hours after bombardment, primarily in cells of the embryonal head (Figures 2C, D). Transiently transformed suspensor cells were more difficult to detect, as their highly vacuolate nature rendered the GFP less detectable. Although suspensor cells occupy a greater volume of the somatic embryo and therefore a greater volume of the embryogenic mass, GFP expression by the suspensor cells within that mass was infrequent. Suspensor cells are a magnitude larger than the embryonal head cells and thus, the nucleus occupies a much smaller proportion of the cell volume than does a nucleus of an embryonal head cell. In fact, the compact nature of the embryonal head makes it more likely that a microprojectile will somewhere penetrate a nucleus, accounting for the higher number of GFP-expressing sites in this region of the somatic embryo.

GFP expression rapidly diminished with time and became very difficult to detect even only 3 days after bombardment. GFP, both wild-type and modified, has been expressed in black spruce (*Picea mariana*), white spruce (*Picea glauca*) and eastern white pine (Tian et al. 1997).

The use of GFP as visual marker for transgenic plants (Leffel et al. 1997) can only be used after overcoming several procedural constraints. First, samples may not be directly viewed for GFP while contained in plastic (petri dish) vessels, since the plastic reduces the intensity of the effective excitatory wavelengths. Second, few fluorescent microscopes have sufficient stage clearance for observation of undisturbed transformed cultures. In our laboratory, the samples had to be flattened in order to spread the cells for visualization which disrupted the culture integrity and any gross morphological structures

were disaggregated. Third, the GFP fluorescence was easily detectable under 100X magnification but was less discernible at 40X. The higher magnification greatly lengthened the time for collecting data when compared to assaying for GUS expression which can be visualized under lower magnifications.

However, the detection of GFP is more expedient and inexpensive than testing for GUS expression, since the latter requires both an overnight incubation as well as an expensive substrate. If GFP expression can be maintained at adequately high levels to be distinguishable from the autofluorescence, it will be an appropriate selectable marker for eastern white pine transformation.

Distinctive expression in coniferous tissues

As noted by Walter et al. (1994) tissues of *Pinus radiata* exhibit the uncommon property of responding to both monocot and dicot promoters. Expression in eastern white pine embryogenic cultures follows a similar pattern, as strong expression of GUS was found when driven by both a monocot-optimized (pMON752 – 35S-35S + *adh1* intron) and a dicot-optimized (pBI426 – 35S-35S + AMV enhancer) promoter. In addition, the plant viral promoters (35S, *amt*) can also be as effective as the angiosperm promoters, suggesting that conifers can serve as tools for deciphering the control and specificity of transcription.

Apparently the conifer transcription/translation machinery may be more tolerant of variability, to still allow expression of introduced genes. For example, the presence of a 5′ intron (Figure 1), characteristic of monocot constructs, is apparently not a required element or a regulatory factor for conifer translation. This is evident from the higher expression of the *Ubi1*-driven GUS construct (pAHC25) than the *adh1*-driven one (pAI_1GUS_nDy10s), although both constructs have a similarly positioned intron. Conversely, the presence of the intron did not disrupt expression, as the 35S-35S + *adh1* intron-driven construct (pMON752) generated high numbers of GUS foci as did the 35S-35S-driven construct (pMON18350), which lacked such an intron. Promoter deletion analysis could provide additional clues to sequences critical for control of expression in conifer systems and, possibly, for elements universal to the transcription/translation process.

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